Inflight Central Nervous System Tissue Fixation for Ultrastructural Studies

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RATIONALE

The cerebellum is a brain structure critical for coordination, timing muscle contractions during movement, and motor learning. Anatomical studies have shown that neurons in the cerebellum are structurally modifiable. These investigations have demonstrated changes in neuron shape, as well as in the three-dimensional structure of the specialized contacts between nerve cells (synapses) as a result of altered behavioral experiences. Physiological studies have provided some insight into the functional significance of those structural modifications in the performance of motor tasks. The objective of our Neurolab experiment was to evaluate the fine structure of the cerebellum at several time points during and following spaceflight in order to identify architectural alterations in the rat cerebellum that correlate with the adaptation to microgravity, and re-adaptation to Earth's gravity. This required the ability to preserve (fix) the tissue acquired in space so that it could be analyzed after landing.

A major technical challenge that faced our experiment was determining the optimal fixation strategy for the brain tissue collected during flight, while ensuring the safety of the crew and the Shuttle. Tissue fixation is necessary in order to preserve the integrity of the biological specimens intended for future study. The essence of good tissue fixation for anatomical studies is preservation of fine ultrastructural detail (the structure of the individual nerve cells and their connections). This detail is usually seen with electron microscopes. Most laboratories conducting similar studies of the central nervous system (CNS) inject chemical preservatives (aldehydes, most commonly paraformaldehyde and glutaraldehyde) through the blood vessels of the brain in a process called perfusion fixation. Although perfusion fixation is the method of choice for ultrastructural investigations, the method requires relatively large volumes of these toxic chemicals, which may escape into the closed Shuttle environment and harm the crew. Alternative procedures such as rapid specimen freezing, which can be used in some types of ground research, were not possible for the specific goals of this experiment because they disrupt brain ultrastructure. In addition, microwave fixation was not possible since microwaves may interfere with the Shuttle's operating systems. As a result, a series of ground studies was conducted to develop and validate an immersion fixation procedure for spaceflight, in which tissue specimens are harvested and placed into vials of liquid fixative that then diffuses into the tissue over time. This approach tends to provide poorer-quality tissue fixation than that obtained by perfusion fixation, since pathologic degradation of the specimen usually occurs during the diffusion-dependent time interval between euthanasia and actual specimen fixation. Nevertheless, this technique was attempted to avoid the possible release of dangerous chemicals into the Shuttle environment and to provide a method that could be used successfully in space.

PROCEDURE DESCRIPTION

The first ground study was designed to establish the optimal time period required for immersion fixation of the cerebellum. The specific objective of this experiment was comparing the ultrastructural tissue preservation at 18 days vs. 30 days of immersion fixation. Initially, 12 rats were sacrificed. The brains were removed, placed in vials containing ice-cold (4°C) paraformaldehyde (PF) fixative, and refrigerated. After 18 days, half the specimens were removed from their vials and sectioned as they were to be cut for the flight experiment. These slices of cerebellum were subsequently processed and then cut into 50-nm sections for electron microscopic analysis. The second set of brains remained in fixative for 30 days, and was then treated in the same way (see Table 1).

Twenty electron micrographs were taken of each brain. The number of holes in the tissue, the sizes of the holes, the total area, and the percent area of holes per electron micrograph and per brain were determined in coded specimens in order to apply an objective quantitative measure to evaluate the condition of each cerebellum. As indicated in Table 1, these data were used to rank order the best six conditions. In general, this quantitative assessment indicated that 5.59% of the total area of sampled brain was occupied by pathologic holes attributable to poor fixation in the 30-day immersion-fixed specimens, in comparison with 2.54% in the 18-day immersion-fixed tissue. These quantitative results were substantiated by qualitative observations of poor tissue preservation and pathologic degradation in the 30-day fixed samples not observed in the 18-day fixed tissue (see Figures 1 and 2). The results demonstrated clearly that 18 days of immersion fixation provided far better tissue quality than 30 days of fixation. They also reinforced the importance of rapid brain harvesting.

The second ground study was designed to determine the optimal fixation sequence for immunocytochemical studies of immersion-fixed cerebella. Immunocytochemistry is a method that can be used to identify specific molecules associated with brain cells. Often, tissue preservation requirements for immunocytochemistry conducted in conjunction with electron microscopy are different from those for electron microscopy alone. Since one aim of the Neurolab experiment was identifying changes in cellular molecules during and following spaceflight, a technical study to compare ultrastructural tissue preservation and immunocytochemical staining obtained with different 18-day immersion fixation strategies was required. Initially, four sets of conditions were evaluated, as indicated in Table 2.

The brains were obtained using the same procedure as for the first experiment. After 18 days in the immersion solution, the specimens were removed from their vials, and processed for ultrastructural (electron microscopy) analysis or for immunocytochemistry to visualize the presence of GABA, a neurotransmitter used by many cells in the cerebellum. Thin

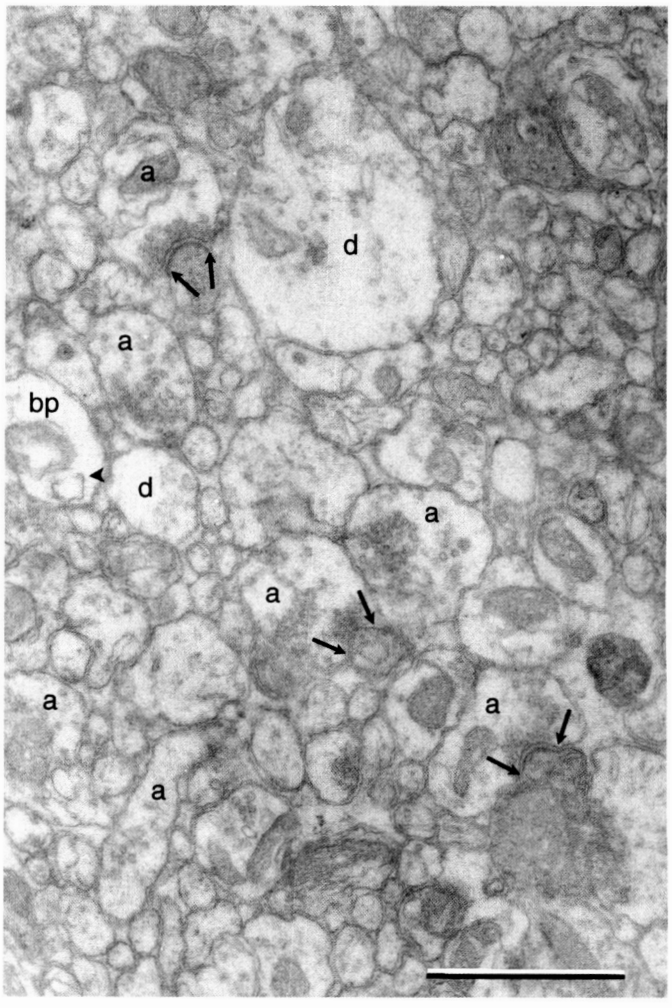

Figure 1. Electron micrograph from a rat cerebellum immersion-fixed in 4% paraformaldehyde for 18 days. The ultrastructure preservation is acceptable, with few bloated profiles (bp), torn membranes (arrowhead), or signs of pathologic degradation of the tissue. Well-preserved axon terminals (a) containing clusters of synaptic vesicles often formed synaptic contacts (arrows) with adjacent dendrites. Other large dendritic cross-sections (d) are also well fixed. Scale bar: 1 μm.

Table 1. The effect of fixative composition, duration of immersion fixation, and brain extraction time on ultrastructural tissue preservation.

Rat #	Fixative	Duration in fixative	Brain extraction time	Results
1	2% PF	30 days immersion	2.5 min.	Rejected.
2	2% PF	18 days immersion	2 min.	**Ranked #4.**
3	4% PF	30 days immersion	2 min.	Rejected.
4	2% PF	18 days immersion	2 min.	**Ranked #5.**
5	2% PF	30 days immersion	3.5 min.	Rejected.
6	4% PF	18 days immersion	1.5 min	**Ranked #1.**
7	2% PF	30 days immersion	1.5 min.	Rejected
8	2% PF	18 days immersion	1.5 min.	**Ranked #2.**
9	4% PF	30 days immersion	2 min.	Rejected.
10	2% PF	18 days immersion	2 min.	**Ranked #6.**
11	2% PF	30 days immersion	1.5 min.	Rejected.
12	4% PF	18 days immersion	2 min.	**Ranked #3.**

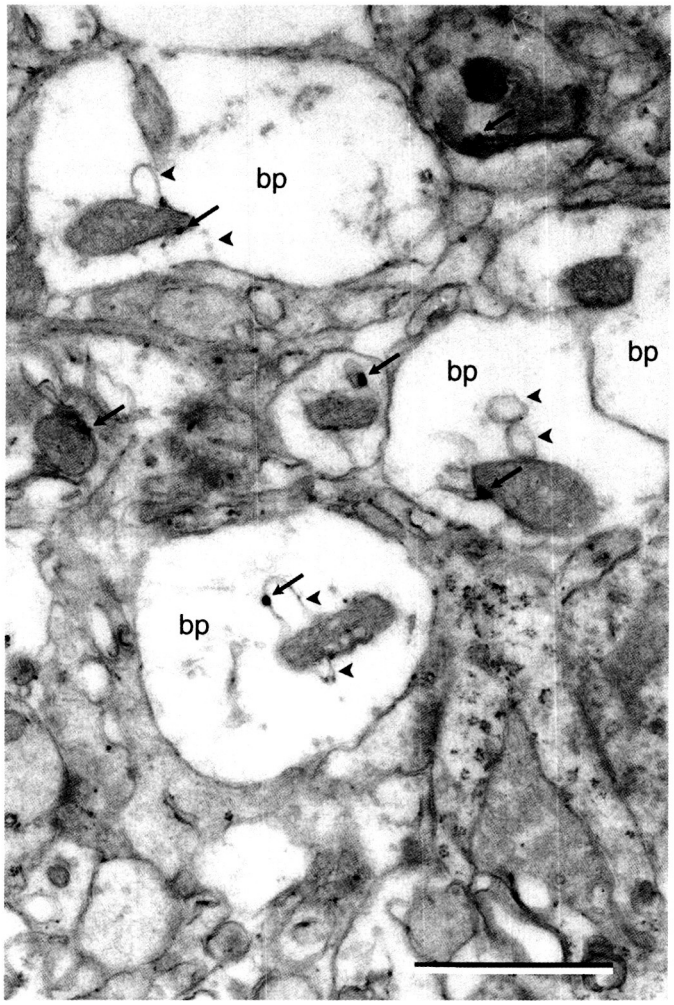

Figure 2. Electron micrograph from a rat cerebellum immersion-fixed in 4% paraformaldehyde for 30 days. The ultrastructure preservation is unacceptable, with many bloated profiles (bp), torn membranes (arrowheads), and signs of pathologic degradation of the tissue (arrows). Scale bar: 1 μm.

sections were evaluated quantitatively for tissue condition, and qualitatively for immunoreactivity in known GABAergic neurons. The quantitative assessment of tissue quality indicated that significantly better preservation of the brain tissue was obtained using the higher concentration of PF fixative for the extended exposure period. However, little immunocytochemical staining was observed. As a result, we concluded that (1) the tissue must remain in fixative (not in buffer) for the 18 days; (2) improved ultrastructure is obtained with 4%, rather than 2%, PF; but (3) a lower concentration of glutaraldehyde should be utilized in the fixative to improve immunocytochemical staining.

On the basis of these results, three additional fixation sequences were evaluated for use with each of two possible immunocytochemical staining methods (see Table 3). In particular, for three additional fixation protocols, half of the brain tissue was used for "pre-embedding" immunocytochemistry, and the other half for "post-embedding" studies. Using the former technique, the immunocytochemical procedure is conducted on brain slices before they are prepared for electron microscopy. Using the latter technique, the immunocytochemistry is performed directly on thin sections that have already been prepared and then cut for electron microscopic analysis. In general, the former technique provides larger sample sizes, whereas the latter approach carries the advantage of better tissue quality. Having evaluated these two methods with three additional fixation sequences, it was concluded that the optimal fixation conditions for the Neurolab experiment were: 4% PF with 0.1% glutaraldehyde for 45 minutes, then 4% PF for 18 days, all at 4°C. We further concluded that better tissue quality and staining were obtained when the immunocytochemistry portion of the study was conducted after the brain slices were prepared for electron microscopy ("post-embedding" immunostaining).

The last fixation experiment was performed in order to verify that the fixatives selected for the Neurolab experiment could withstand the storage requirements for the flight. The fixative solutions were to be prepared on the ground, two days prior to Shuttle launch. Since brains were to be collected for this experiment on flight days (FDs) two and 14, it was criti-

Table 2. The effect of fixation conditions on immunocytochemical tissue staining.

Rat #	Fixation conditions	Tissue treatment	Evaluation of staining and fixation
1	2% PF/0.35% glut for 45 min 2% PF for 18 days	ICC	Little ICC staining, mediocre tissue fixation.
2	2% PF/0.35% glut for 45 min 2% PF overnight PBS for 18 days	ICC	Little staining, worst fixation.
3	4% PF/0.35% glut for 45 min 4% PF for 18 days	ICC	Little ICC staining, good tissue fixation.
4	4% PF/0.35% glut for 45 min 4% PF overnight PBS for 18 days	ICC	Little staining, poor fixation.
5	2% PF/0.35% glut for 45 min 2% PF for 18 days	ICC	Little ICC staining, mediocre tissue fixation.
6	2% PF/0.35% glut for 45 min 2% PF overnight PBS for 18 days	ICC	Little staining, worst fixation.
7	4% PF/0.35% glut for 45 min 4% PF for 18 days	ICC	Little ICC staining, good tissue fixation.
8	4% PF/0.35% glut for 45 min 4% PF overnight PBS for 18 days	ICC	Little staining, poor fixation.
9	2% PF/0.35% glut for 45 min 2% PF for 18 days	EM-stereology	Ranked #2
10	2% PF/0.35% glut for 45 min 2% PF overnight PBS for 18 days	EM-stereology	Ranked #4
11	4% PF/0.35% glut for 45 min 4% PF for 18 days	EM-stereology	Ranked #1
12	4% PF/0.35% glut for 45 min 4% PF overnight PBS for 18 days	EM-stereology	Ranked #3

Abbreviations: electron microscopy (EM), glutaraldehyde (glut), immunocytochemistry (ICC), paraformaldehyde (PF), phosphate-buffered saline (PBS).

Table 3. The effect of glutaraldehyde concentration on immunocytochemical tissue staining.

Rat #	Fixation conditions	Tissue treatment	Staining and fixation evaluation
1	4% PF/0.05% glut for 45 min 4% PF for 18 days	Pre-ICC	Mediocre fixation, mediocre staining
2	4% PF/0.1% glut for 45 min 4% PF for 18 days	Pre-ICC	Acceptable fixation, mediocre staining
3	4% PF/0.25% glut for 45 min 4% PF for 18 days	Pre-ICC	Acceptable fixation, little staining
4	4% PF/0.05% glut for 45 min 4% PF for 18 days	Post-ICC	Mediocre fixation, acceptable staining
5	4% PF/0.1% glut for 45 min 4% PF for 18 days	Post-ICC	Acceptable fixation, acceptable staining
6	4% PF/0.25% glut for 45 min 4% PF for 18 days	Post-ICC	Acceptable fixation, little staining
7	4% PF/0.05% glut for 45 min 4% PF for 18 days	Pre-ICC	Mediocre fixation, mediocre staining
8	4% PF/0.1% glut for 45 min 4% PF for 18 days	Pre-ICC	Acceptable fixation, mediocre staining
9	4% PF/0.25% glut for 45 min 4% PF for 18 days	Pre-ICC	Acceptable fixation, little staining
10	4% PF/0.05% glut for 45 min 4% PF for 18 days	Post-ICC	Mediocre fixation, acceptable staining
11	4% PF/0.1% glut for 45 min 4% PF for 18 days	Post-ICC	Acceptable fixation, acceptable staining
12	4% PF/0.25% glut for 45 min 4% PF for 18 days	Post-ICC	Acceptable fixation, little staining

Abbreviations: glutaraldehyde (glut), paraformaldehyde (PF), pre-embedding immunocytochemistry (pre-ICC), post-embedding immunocytochemistry (post-ICC).

cal to determine whether the fixatives remained potent for the 16 days between solution preparation and tissue immersion for the FD14 brains, and then for the 18 additional days of immersion fixation. For this experiment, the ultrastructural tissue preservation and immunocytochemical staining for GABA were compared in brains fixed using the protocol developed in the previous experiment. One half of the brain was placed in freshly prepared fixative (4% PF with 0.1% glutaraldehyde in 0.1M phosphate buffer for 45 minutes, then 4% PF for 18 days). The other half-brain was placed in fixatives (same solutions) prepared 16 days before euthanasia. After 18 days of immersion fixation, the cerebella were cut into slices and then processed for electron microscopy. We found no differences in the ultrastructural tissue preservation of fresh vs. stored fixative.

APPLICATION

The fixation studies described above were verified in an integrated experiment verification test held at NASA/Ames Research Center (ARC). This test involved three experimental groups that were representative of the subject groups planned for the Neurolab Shuttle mission. They included a flight group, a vivarium group, and a hypergravity group. To simulate hypergravity at two times the Earth's gravitational force, this latter group was placed in the 24-foot centrifuge facility at ARC. The 24-foot centrifuge consists of a central vertical shaft spindle driven by a 25-horsepower motor. Attached to the top of the spindle, approximately six feet from the ground, are 10 radial arms. Each arm holds two enclosures 23.5 in. high×39.5 in. wide ×22 in. deep. The centrifuge was set at 19.99 RPM to create a two-G environment at the floor of the animal cages. As with the planned Shuttle mission, each experimental group above had four sets of subjects: FD2 (N=4), FD14 (N=9), recovery (R) day+1 (N 4), and R+13 (N=7). The cerebellum from each of these 72 rats was immersion-fixed according to the protocol developed previously, and then sectioned and processed for electron microscopy. Tissue analysis verified that the fixation protocol developed for flight could provide acceptable tissue quality for conducting the electron microscopic and immunocytochemical studies proposed for the Neurolab experiment. In fact, most of the brain tissue subsequently obtained from the Neurolab mission proved to be acceptable for ultrastructural study. Observations of cerebellar tissue from FD2 rats are included elsewhere in this volume, and illustrate the utility of this fixation approach.

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